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STAT3 activation impairs the stability of Th9 cells

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Abstract

Th9 cells regulate multiple immune responses including immunity to pathogens and tumors, allergic inflammation, and autoimmunity. Despite ongoing research into Th9 development and function, little is known about the stability of the Th9 phenotype. In this report we demonstrate that IL-9 production is progressively lost in Th9 cultures over several rounds of differentiation. The loss of IL-9 is not due to an outgrowth of cells that do not secrete IL-9, as purified IL-9 secretors demonstrate the same loss of IL-9 in subsequent rounds of differentiation. The loss of IL-9 production correlates with increases in phospho-STAT3 levels within the cell, and the production of IL-10. STAT3-deficient Th9 cells have increased IL-9 production that is maintained for longer in culture than IL-9 in control cultures. IL-10 is responsible for STAT3 activation during the first round of differentiation, and contributes to instability in subsequent rounds of culture. Together, our results indicate that environmental cues dictate the instability of the Th9 phenotype, and suggest approaches to enhance Th9 activity in beneficial immune responses.

Introduction

The specialized ability of CD4 T helper cells to mediate distinct aspects of pathogen immunity is based on the acquisition of restricted cytokine-secreting profiles. The acquisition requires responsiveness to cytokine environments and the differentiation of cells into subsets that express transcription factors and cytokines that are associated with a specific lineage. In the current nomenclature, Th1 cells express T-bet and IFNγ, Th2 cells express GATA3 and IL-4, and Th17 cells express RORγt and IL-17 (1). Yet, these differentiated states are not necessarily terminal or stable. In specific cytokine environments, Th2 and Th17 cells can acquire Th1 patterns of gene expression and cytokine production (2–

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7). At the molecular level, this plasticity is related to the establishment of poised chromatin states at the loci of transcription factors that are key regulators of these differentiation processes (5, 8). The biological necessity for Th plasticity is not entirely clear, but likely includes increased ability of a T cell population to respond in an evolving inflammatory milieu, but also potentially unique characteristics of Th cells that transit from one state to another.

The Th9 subset of Th cells is among the most recently described. Th9 cells secrete IL-9 as a hallmark cytokine and require transcription factors for differentiation that include PU.1, IRF4, BATF, GATA3, ETV5 (9, 10) and potentially others. Th9 cells promote allergic inflammation in the lung, and parasite immunity, are linked to food allergy in patients, and are implicated in the development of ulcerative colitis in patients and mouse models. The precise mechanisms of Th9 functions are not entirely clear, but in several of these immune responses, outcomes of Th9 responses are likely due to their ability to promote Th2 responses and mast cell accumulation in target tissues.

The plasticity of Th9 cells is still not well understood. Initial reports suggested that IL-9 production was only transient in vitro, and adoptively transferred Th9 cells mediated autoimmune inflammation that was IFNγ and not IL-9 dependent (11, 12). Yet, in asthma models, adoptively transferred Th9 cells retain the ability to promote inflammation and cause mast cell accumulation in an IL-9-dependent manner, and cytokines such as TSLP help to maintain cytokine production (13–16). Moreover, in patients, allergen-specific recall responses can be observed (17–19). Together, these studies suggest that in an appropriate environment, Th9 cells can maintain lineage-specific cytokine production.

The acquisition of lineage-specific cytokine production requires chromatin modification and locus remodeling that occurs over days to weeks (20, 21). Early reports that studied the stability of Th cell phenotypes examined cytokine production after 2–3 rounds of 5–7 day differentiation culture periods (22). In contrast, many studies of Th9 cells have examined cytokine production after limited in vitro culture, a time when the *Il9* locus is activated, but not necessarily programmed. In these studies, we sought to examine Th9 stability, and used optimized culture conditions and *Il9*-reporter mice to define the ability of IL-9-secreting T cells to maintain their differentiated phenotype.

Materials and Methods

Mice

C57BL/6 mice were bred in house at the IU School of Medicine, Indianapolis, IN, USA or purchased from Harlan Bioproducts (Indianapolis, IN). *Il9*-Citrine reporter mice (23), and *Stat3* conditional mutant mice (24) have been previously described. All mice were used with the approval of the Indiana University Institutional Animal Care and Use Committee.

Naïve CD4 T cell isolation and in vitro culture

$CD4^+$ $CD62L^+$ T cells were isolated from the spleens of the indicated mice using magnetic separation following the supplier's protocol (Miltenyi Biotec, Auburn, CA). Cells were cultured at 10^6 cells per ml in complete RPMI on plates coated with anti-CD3 and soluble

anti-CD28 as previously described (25). Further, cells cultured under Th9 conditions were supplemented with human TGF-β1 (2 ng/ml), IL-4 (20 ng/ml), and anti-IFN-γ (10 μg/ml). In some experiments, IL-10 signaling during differentiation was blocked using IL-10 and IL-10R neutralizing antibodies (respectively, 10μg/ml, BioXcell, JES5-2A5 or 20 μg/mL, BioXcell, 1B1.3A). Th2 cells were cultured identically to Th9 cells, but in 10 ng/ml IL-4 and in the absence of TGF-β1. Th1 cells were cultured with murine IL-12 (10ng/mL), anti-IL-4 (11B11; 10 μg/mL), and human IL-2 (50 U/mL). Th17 cells were cultured with murine IL-6 (100 ng/mL), human TGF-β1 (2 ng/ml), murine IL-1β (10 ng/mL), IL-23 (10 ng/mL), anti-IFNγ (XMG; 20 μg/mL), anti-IL-4 (11B11; 10 μg/mL), anti-IL-2 (10 μg/mL). After the initial 3 days in culture cells were removed from anti-CD3-coated plates and expanded with three volumes of fresh media containing the same concentrations of IL-4 and TGF-β1 and neutralizing antibodies. For the second and third rounds of culture, cells were cultured and expanded as per the initial round of differentiation. Where described, citrine^{+} T cells from *Il9*-Citrine reporter mice were sorted using a BD FACS Aria in the IUPUI flow cytometry core facility.

Intracellular cytokine staining (ICS) and STAT staining

After the indicated times in culture, the frequency of cytokine-producing T cells was determined by ICS. Briefly, 0.5–1.0x10^{6} cells were stimulated in media containing PMA and ionomycin or plate bound anti-CD3 as previously described (25). After 2–3 hours monensin (2μM) was added to stimulated cells and 3 hours later, cells were stained with a fixable viability dye (eBioscience) and fixed with 4% formaldehyde at room temperature for 10 minutes. After fixation, cells were permeabilized with permeabilization buffer (eBioscience) and stained for intracellular cytokines (IL-4: 11B11 [BioLegend], IL-9: RM9A4 [BioLegend], IL-10: JES5-16E3 [BioLegend], and IL-17A: eBio17B7 [eBioscience]) in the same buffer.

Staining for intracellular STATs was done after fixation with 4% formaldyhyde, as above, and permeabilization with cold 100% methanol. Cells were subsequently washed with PBS and stained with flourochrome-labeled antibodies to total STAT3, pSTAT3(Y705) and pSTAT5(Y694) (all from BD Biosciences).

Real-time PCR

RNA was harvested from Th9 cells at the indicated time points in Trizol reagent (Life Technologies). cDNA was produced by reverse transcribing mRNA via the manufacturer's direction (Invitrogen). Real-time PCR was carried out with TaqMan primers (Life Technologies) using a 7500 Fast-PCR machine (Life Technologies).

Statistics and data analysis

All statistics were done using Prism software version 7 (GraphPad) using Student *t* test unless otherwise stated. Flow cytometry data was collected using an Attune flow cytometer (Life Technologies) and was analyzed using FloJo version 10 (Tree Star).

Results

Stability of IL-9-secreting T cells

To begin to address the ability of Th9 cells to maintain cytokine production over time, we initiated long-term cultures. As described in Methods and shown in Fig. 1A, naïve CD4 T cells from C57BL/6 mice were activated with anti-CD3 and anti-CD28 in the presence of IL-4 and TGFβ1. After three days, cells were expanded in the presence of additional cytokines. After five days in culture, cells were either tested for cytokine production by intracellular cytokine staining, or cultured under identical conditions for another 1–2 rounds of 5 day differentiation, as indicated. In this report, we will refer to cells after one round of differentiation as “round 1” (R1), “round 2” (R2), or “round 3” (R3).

We observed significant populations of IL-9 secreting T cells in R1D5 cultures, but percentages of cells that were producing IL-9 progressively diminished over culture from R1 to R2 to R3 (Fig. 1B–C). We also observed the induction of expression of transcription factors required for IL-9 production including GATA3, IRF4 and BATF, from naïve cells to R1, but expression then fell between R1 and R2 (Fig. 1D).

Th9 cells also produce IL-10, and we have previously suggested that the IL-9 and IL-10 producing populations in Th9 cultures are distinct (26). In contrast to the results observed with IL-9, production of IL-10 increased over time (Fig. 1B–C). Indeed, there was a strong inverse correlation between the numbers of IL-10 and IL-9-secreting cells in Th9 cultures (Fig. 1C).

Isolation of IL-9-secreting T cells

One of the limitations of the approach above is that Th9 cultures are a mosaic of cells, even when there is a predominance of the IL-9-secreting phenotype. As such, one possible interpretation of these results is that the non-IL-9-secereting cells outgrow the IL-9-secreting T cells in culture. To directly address this concern, we utilized recently generated *Il9* reporter mice (23). These mice were constructed with Citrine (a YFP derivative) knocked into the *Il9* locus. To provide a more detailed analysis of the fidelity of the *Il9* reporter expression, we differentiated wild type, *Il9*-Citrine heterozygous ($Il9^{+/Cit}$), and $Il9^{Cit/Cit}$ mice under Th1, Th2, Th9, and Th17 polarizing conditions. We observed citrine expression in $Il9^{+/Cit}$ and $Il9^{Cit/Cit}$ Th9 cultures, and a small percentage of cells that were citrine positive in Th2 cultures, but essentially no positive cells in Th1 or Th17 cultures (Fig. 2A–B). In the $Il9^{+/Cit}$ Th9 cultures, about 75% of the Citrine positive cells are also IL-9-positive. However, there is a large population of cells that are IL-9 positive and Citrine negative (Fig. 2A). This could result from slower translation of Citrine than IL-9, but might also result from the $Il9^{Cit}$ allele being transcribed at a lower efficiency than the wild type IL-9 allele. The percentage of citrine positive cells in the $Il9^{Cit/Cit}$ cultures is only about a third of the IL-9 positive cells in wild type Th9 cultures. Together, these results suggest that the $Il9^{Cit}$ allele faithfully reports for IL-9 expression, but that it likely underrepresents the percentage of cells that are expressing IL-9.

We then used the $Il9^{+/Cit}$ mice to address the question of competition among populations. Naïve CD4 T cells from $Il9^{+/Cit}$ mice were cultured under Th9 conditions for five days and

sorted into Citrine-positive and –negative populations (Fig. 3A). The sorted cells (R1) were predominantly IL-9-positive and had minimal expression of IL-4, IL-10, or IL-17A (Fig. 3A–D). Sorted cells were then cultured for an additional five-day culture. Similar to the results from bulk cultures, IL-9 production, measured both as Citrine and IL-9, decreased dramatically between R1 and R2 (Fig. 3A–C). However, cells did not display plasticity to a specific phenotype. Citrine-positive and –negative cells were predominantly IL-10 secretors but had smaller populations of IL-4-positive, and IL-17A-positive cells (Fig. 3A and C).

We extended these analyses by examining the segregation of expression of other genes associated with the Th9 genetic program. As expected *Il9* mRNA was enriched in Citrine-positive cells, as were *Irf4* and *Il1rn* (Fig. 3D). *Batf* was not significantly different between Citrine-positive and –negative, and *Foxp3* was enriched in Citrine-negative cells (Fig. 3D). However, after R2, there was a significant drop in expression of *Irf4*, *Batf*, and *Il1rn* in both Citrine-positive and –negative populations, and increases in *Foxp3*, particularly in the Citrine-negative population (Fig. 3D). Thus, Th9 cells do not maintain a Th9 phenotype even after sorting for enrichment of IL-9 expression.

STAT3 represses IL-9 in long-term Th9 cultures

We then speculated that the loss of IL-9 secretion over time is not due to a stochastic loss of locus expression, but rather due to signals that repressed the *Il9* locus. Notably, we had observed induction of IL-10 in Th9 cultures over time (Fig. 1 and 3). As IL-10 is both an activator and a target of STAT3 (27), and we have recently shown that STAT3 is a negative regulator of the Th9 phenotype (25), we examined STAT3 activation in long-term Th9 cultures. We observed that the presence of pSTAT3 within Th9 cells was inversely correlated with IL-9 (Fig. 4A–B), similar to what we observed between IL-9 and IL-10 (Fig. 1). We also observed a significant increase ($p<0.05$) in both the geometric mean fluorescence intensity (gMFI) and % of cells expressing total STAT3 protein in R2 as compared to R1, suggesting an increase in both pSTAT3 and total STAT3 protein as IL-9 production decreases (Fig. 4C). Consistent with this, increases in pSTAT3 paralleled increases in IL-10 (Fig. 4D). We then tested the expression of additional STAT3 target genes. Similar to the induction of *Il10*, we observed increases in *Il24, Il17a* and *Il21* (Fig. 4E). Thus, diminished IL-9 production was coincident with the induction of a STAT3 target signature.

We have previously shown that STAT3 limits IL-2 production as well as STAT5 activation by Th9 cells during the first round of differentiation (25). As accumulation of total STAT3 and pSTAT3 correlated with decreased Th9 stability (Fig. 4A–D), we questioned whether there was also a corresponding decrease in IL-2 production or STAT5 activation over two rounds of Th9 differentiation. There was a significant decrease (~5–6-fold, $p<0.05$) in the capacity of cells to produce IL-2 from the first to the second round of Th9 culture. However, there was little difference in the amount of intracellular pSTAT5 at these time points (data not shown). As IL-2 is required for Th9 differentiation (28), we reasoned that the lack of IL-2 production between R1 and R2 might play a role in the decreased capacity of these cells to produce IL-9 at R2. We therefore added exogenous recombinant human IL-2 to these cultures at the initiation of R2 and examined their capacity to produce IL-2 and IL-9 at the end of R2. Interestingly, the addition of IL-2 to these cultures had no effect on IL-2 or IL-9

production (Fig. 4F) or the amount of intracellular pSTAT5 (>50% of cells were pSTAT5^{+}, data not shown). Therefore, it is unlikely that the instability of the Th9 phenotype is solely due to an inability to produce IL-2.

To directly test the function of STAT3 in Th9 instability, we differentiated *Stat3* fl/fl CD4-Cre naïve T cells or littermate Cre-negative controls under Th9 conditions. As we demonstrated previously, there were increased percentages of IL-9-positive cells and increased intensity of IL-9 staining within the IL-9-expressing cells (Fig. 5A–B)(25). Importantly, at R2, when IL-9 had dropped significantly in the control cultures, IL-9 production was maintained in STAT3-deficient cultures, and the increases in IL-10 were more modest (Fig. 5A–C). However, by R3, IL-9 production had dropped to low levels, similar to those observed in control cultures, even in the absence of STAT3 (Fig. 5C).

IL-10 impairs IL-9 production and Th9 stability

Since STAT3 had a negative impact on IL-9 production during R2, we tested whether endogenous IL-10 impaired R2 differentiation. We differentiated *Il9*$^{+/Cit}$ naïve T cells under standard Th9 conditions for R1 and sorted Citrine-positive cells. During R2 cells were then cultured under Th9 conditions in the presence or absence of anti-IL-10R. Blocking IL-10R was able to maintain IL-9 production in a significantly greater proportion of cells than in cultures without IL-10R blockade, although there was still a significant decrease compared to R1 IL-9 production (Fig. 6A–B), and similar results were obtained using Th9 cells derived from IL-10R-deficient mice (data not shown). Anti-IL-10R was also able to minimize the increase in the IL-10- and IL-4-secreting populations, and had modest effects on increasing IL-17A (Fig. 6A–B). We further observed that addition of antibodies to IL-6 and IL-21, other STAT3-activating cytokines, did not impair the loss of IL-9 during R2 (data not shown). These results suggest that IL-10 is a component of STAT3-mediated instability of Th9 cells and that blockade can extend the maintenance of the Th9 genetic program.

Discussion

IL-9-secreting T cells mediate a number of immune responses, but the factors that mediate the stability of the phenotype are still not well defined. In this report we have demonstrated that Th9 cells in long-term culture do not maintain IL-9 production over multiple rounds of differentiation. STAT3 is a major mediator of limiting IL-9 production over the course of the first two rounds of differentiation. Thus, we have defined a phased loss of IL-9 production due to a series of environmental cues. In the first round of differentiation, an IL-10/STAT3 pathway limits IL-9 production. In the second round, STAT3 remains a major inhibitory factor and blocking IL-10 has modest but significant effects on maintaining IL-9. In contrast, blocking IL-21 does not affect IL-9 production or pSTAT3 levels (data not shown), suggesting that endogenous cytokines in addition to IL-10 and IL-21 limit IL-9 production at this stage. However, loss of IL-9 production during a third round of stimulation becomes STAT3-independent, and suggests there are additional signals that limit the ability of T cells to make IL-9.

At least part of the ability of STAT3 to limit IL-9 production is by decreasing STAT5 activation, and we previously demonstrated that ectopic expression of an active STAT5

overcame STAT3-mediated inhibition of IL-9 production (25). Interestingly, over multiple rounds of culture where we observed elevated pSTAT3 and total STAT3 levels, we also measured undiminished pSTAT5 (data not shown) despite diminished endogenous IL-2 production (Figure 4F). This data was somewhat surprising given our earlier results and suggests that the ability of STAT3 to destabilize the Th9 lineage is unlikely due to regulating STAT5 activation. It also suggests that Th9 cells can produce factors other than IL-2 that activate STAT5 during the differentiation process. Several STAT3-induced cytokines, including IL-10 and IL-21, also activate STAT5 (29, 30). These cytokines are increased over multiple rounds of Th9 differentiation and it is interesting to speculate that these cytokines might activate both STAT3 and STAT5 with the negative effect of STAT3 being dominant.

It is interesting that endogenous factors in Th9 cultures seem to limit the stability of the IL-9-secreting phenotype. This is seen to some extent in other Th culture systems, particularly where IFNγ limits generation of Th2 and Th17 cells, and antibodies that block IFNγ are standard components of the culture system. Anti-IFNγ is also a standard component of Th9 cultures, as it likely has negative effects on IL-9 production (28). We have further shown that addition of antibodies that block IL-10 signaling during the first and second rounds of culture increases production of IL-9. It is still unclear what additional factors mediate activation of STAT3 and loss of IL-9. Our results suggest it is not IL-6, which although potent in its ability to repress IL-9 (25) is not produced by T cells at appreciable amounts, and IL-6R chains are progressively decreased in expression after T cell activation (data not shown). Moreover, blocking IL-6R in vitro had little affect on stability (data not shown). These observations raise the issue of whether instability of Th9 cells in vitro is somewhat an artifact of culture conditions. As noted previously, Th9 cultured in vitro for one round can be adoptively transferred and retain IL-9 production and IL-9-dependent functions in both allergic airway disease and tumor models (13–16, 31, 32). As a whole, these data indicate that conditions found within settings of inflammation are capable of maintaining a stable population IL-9-producing T cells, with tumor environments providing even more stability, but we have yet to identify these conditions in vitro. This is similar to Th17 cells where the in vitro phenotype might be unstable, but memory Th17 cells develop in vivo (2–7, 33). It is possible that an additional cytokine plays a role in maintaining the Th9 phenotype in vivo. Related to that hypothesis, we tested a variety of cytokines for their ability to maintain IL-9 production in vitro including TSLP, IL-25, IL-33, IL-1β, IL-21, and none had a demonstrable effect on IL-9 production during a second round of in vitro differentiation (data not shown). It is still possible that these cytokines have a role for in vivo stability.

Although STAT3 is a negative regulator of Th9 differentiation and stability in murine cells, it is apparent that some STAT3-activating cytokines (IL-6, IL-10 and IL-21) can promote IL-9 production by human Th9 cells (34). The underlying reason for this discrepancy is still unclear, but it is apparent that the IL-9 promoting or suppressing effects of some of these cytokines is highly concentration dependent. For example, addition of low concentrations of murine IL-21 (<10ng/ml) enhanced IL-9 production, whereas higher concentrations (50ng/ml) were suppressive. In contrast, IL-6 was highly suppressive independent of concentration (25). Furthermore, IL-6, even at low doses, induced long-term activation (up to 24 hrs) of STAT3 whereas IL-10 and IL-21 did not (25) and this correlated with the

capacity to suppress Th9 differentiation. Wong et al (34) did not examine dose response to these cytokines or examine the degree of STAT3 activation after cytokine treatment of human Th9 cells, but it is intriguing to speculate that dose responsiveness or capacity for sustained STAT3 activation after cytokine treatment may be different between murine and human cells.

T helper cell plasticity can be divided into two phases, the elimination of the one genetic program, and the activation of another. In Th17 cells, which seem to be among the most plastic Th subsets, there is evidence that they diminish IL-17 production and induce IFNγ production in vitro and in vivo (3, 4, 6, 7, 35, 36). However, the in vivo plasticity might be restricted to specific environments such as in experimental autoimmune encephalomyelitis. In models of pathogen immunity and allergic inflammation, IL-17A/F-secreting cells do not readily take on other cytokine-secreting phenotypes (7, 35). The physiological advantage for plasticity is still not clear. In one respect, it can limit particular types of inflammation by turning off cytokine production, and can allow an immune response to more rapidly change in response to new extracellular cues. However, it has been suggested that cells that transit through differentiation states might take on unique characteristics. In that model, a Th2 cell that arose directly from a naïve T cell might be functionally different from a Th2 cell that arose from a Th9 cell. It will be interesting to explore this, as the tools develop to answer these questions.

In this report we have defined STAT3 as an inhibitory factor in the initial establishment and stability of the Th9 phenotype. Where IL-9 and Th9 responses might be beneficial, it suggests that there might be approaches to increasing activity including blocking IL-10 or other STAT3-activating cytokines, or by targeting STAT3 itself. These results also suggest approaches to examine Th9 stability in vivo and alter the development of Th9 memory responses during the development of allergic diseases and autoimmunity.

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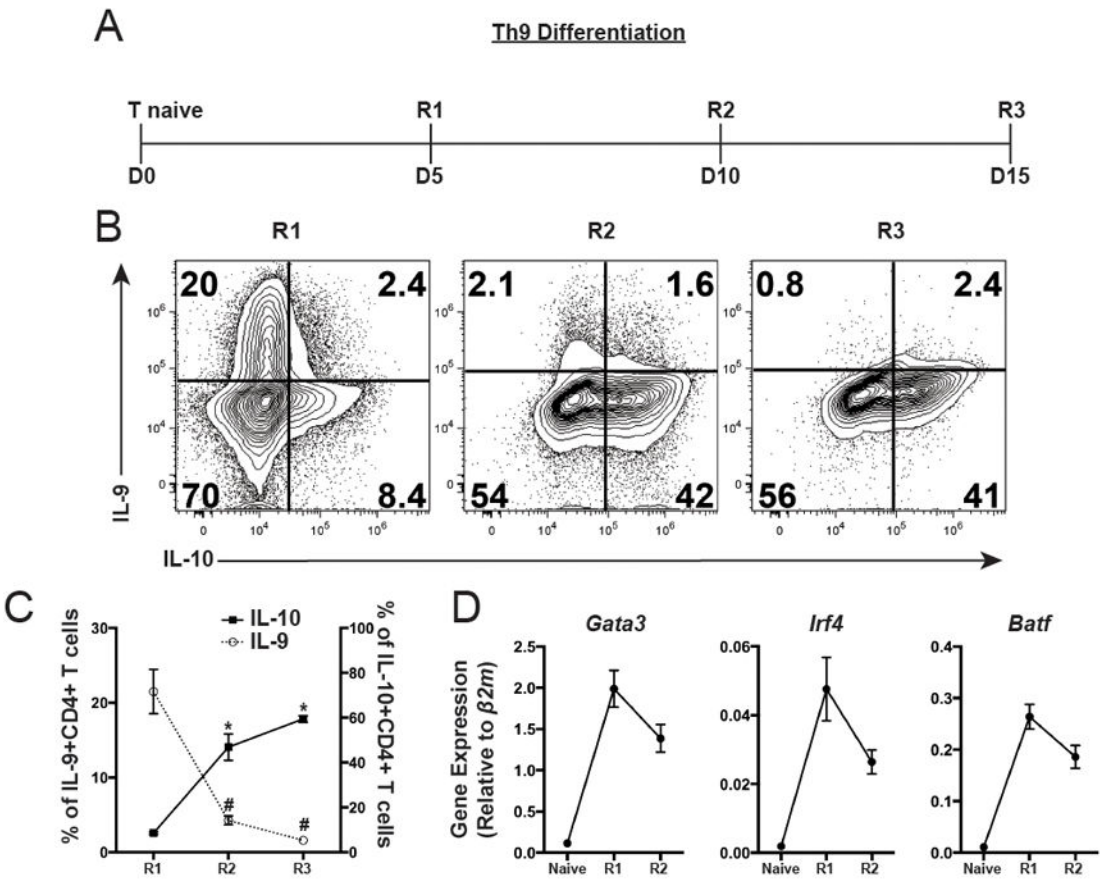

Figure 1.

Loss of IL-9-secreting T cells in Th9 cultures. (A) Long term Th9 cultures were performed with evaluation of cytokines and mRNA at day 5 (D5) of each of the three rounds (R1, R2, and R3) of differentiation. (B) Representative contour plots are shown for intracellular IL-9 and IL-10 staining at R1, R2, and R3 after stimulation with PMA/ionomycin. (C) The frequency of intracellular cytokine^{+} Th9 cells from R1, R2, and R3 quantified from (B). *$p<0.05$, significantly increased as compared to IL-10 at R1. #$p<0.05$, significantly decreased to IL-9 at R1. (D) mRNA collected from unstimulated cells at D0, D5 and D10 was used to determine the expression of transcription factors required for IL-9 production by RT-PCR. These data are representative of 2 individual experiments with 3 individual mice per experiment with data presented in (B) and (C) as the mean ± SEM of three mice in one experiment.

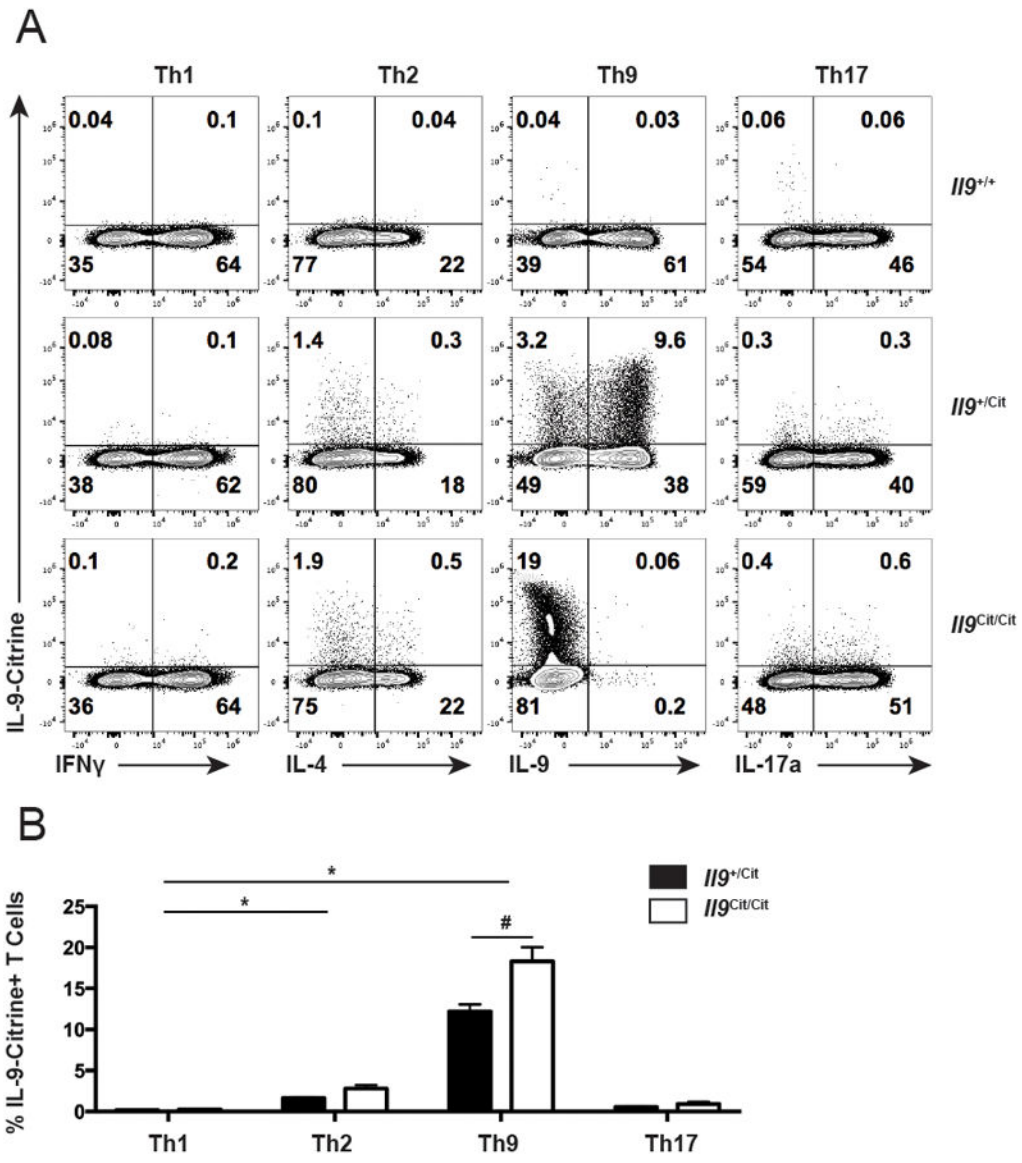

Figure 2.

Characterization of the *Il9*$^{+/Cit}$ reporter activity in T helper subsets. (A) Naïve T cells from *Il9*$^{+/+}$, *Il9*$^{+/Cit}$, *Il9*$^{Cit/Cit}$ mice were cultured in optimized T helper subset conditions. Cultures were harvested after R1 and stimulated prior to intracellular cytokine staining and Citrine florescent protein detection. Th1, Th9, and Th17 culture conditions were stimulated with PMA/ionomycin and monensin. Th2 culture conditions were stimulated with anti-CD3 and monensin. (B) Frequency of Citrine expression in the *Il9*$^{+/Cit}$, *Il9*$^{Cit/Cit}$ mice from T helper subsets on R1 of culture quantified from (A). *p<0.05, significantly increased as compared to Th1 culture conditions. #p<0.05, significantly increased in the *Il9*$^{Cit/Cit}$ compared to *Il9*$^{+/Cit}$. These data are representative of 2 individual experiments with 3 individual mice per experiment with data presented in (B) as the mean ± SEM of three mice in one experiment.

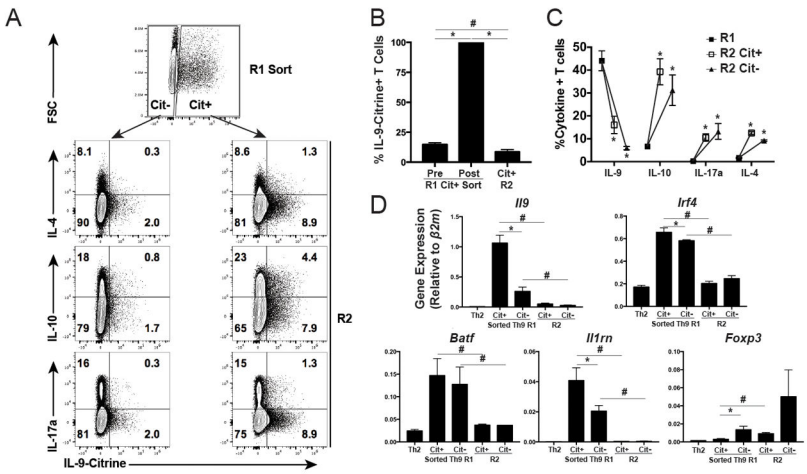

Figure 3.

Isolation and culture of IL-9-secreting T cells. (A) Naïve T cells from *Il9*$^{+/Cit}$ mice were cultured in Th9 conditions. Th9 cells were harvested at the end of R1 and were flow-sorted into Citrine-positive (Cit+) and Citrine-negative (Cit−) populations. Sorted cells were cultured for R2 in standard Th9 conditions and harvested for evaluation of cytokines, Citrine expression, and mRNA. Representative dot plots show the R1 sort of Citrine-negative and -positive populations and the intracellular cytokine expression at R2. (B) Citrine expression prior to and after a Cit+ flow-sort and at the end of R2 of culture. *$p<0.05$, significantly different between pre-sort and post-sort or post-sort and R2. #$p<0.05$, significantly different between pre-sort and R2. (C) The sorted Cit+ population was cultured for R2 in standard Th9 conditions. Cit+ and Cit− gates were used at the end of R2 to examine cytokine expression compared to the original sorted population (R1). *$p<0.05$, significantly different between R1 sorted cells and either the Citrine-positive or -negative gated populations at the end of R2. (D) mRNA collected from unstimulated R1 Th2 cultures, R1 Th9 cultures post-Citrine sort, and sorted Citrine sorted populations cultured for an additional round of standard Th9 conditions (R2). Transcription factors and additional genes related to the Th9 genetic program were assessed by RT-PCR. These data are representative of 3 individual experiments with 3 individual mice per experiment with data presented in (B–D) as the mean ± SEM of three mice in one experiment.

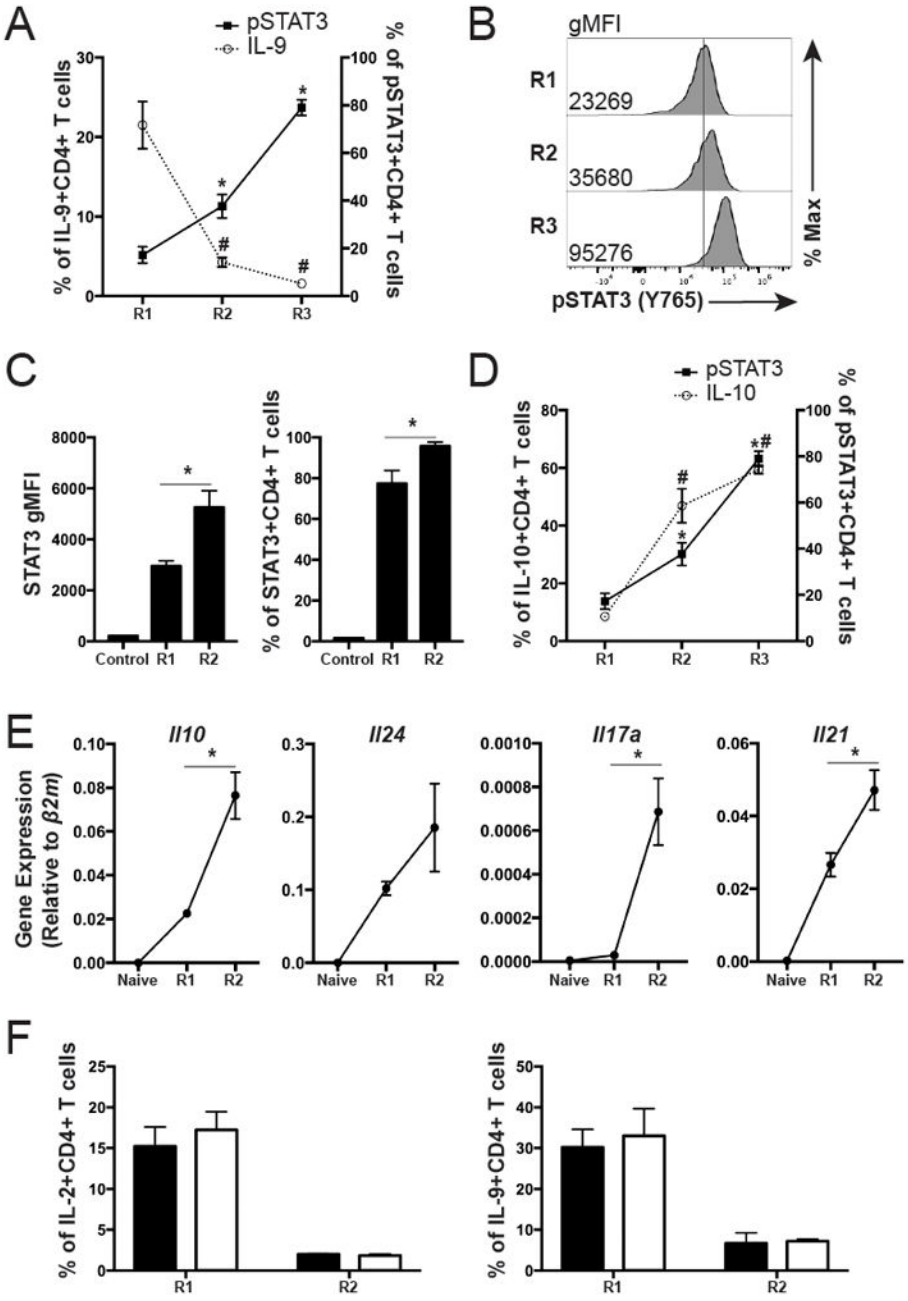

Figure 4.

pSTAT3 is correlated to the loss of IL-9 in long-term Th9 cultures. (A) Frequencies of pSTAT3-positive cells and IL-9-secreting cells over rounds of Th9 culture conditions. *p<0.05, significantly increased as compared to STAT3 phosphorylation at R1. #p<0.05, significantly decreased to IL-9 at R1. (B) Representative histograms depicting and geometric mean fluorescent intensities (gMFI) of pSTAT3 staining over 3 rounds of Th9 differentiation. (C) Total STAT3 gMFI and percent total STAT3+ CD4 T cells. “Control” represents values for an unstained fluorescence minus one (FMO) control. (D) Frequencies of pSTAT3-positive cells (as in (A)) and IL-10-secreting cells over rounds of Th9 culture

conditions. *p<0.05, significantly increased as compared to STAT3 phosphorylation at R1. #p<0.05, significantly increased to IL-10 at R1. (E) mRNA collected from unstimulated cells from naïve, R1 and R2 Th9 cells were used to determine the expression of pSTAT3 target genes by RT-PCR. *p<0.05, significantly increased as compared to from R1 to R2. (F) The frequency of IL-2-positive (left) and IL-9-positive (right) cell when treated, starting at R2 with 20U/ml of rhIL-2 at day 5 and 50U/ml at day 8. These data are representative of 2 individual experiments with 3 individual mice per experiment with data presented as the mean ± SEM of three mice in one experiment.

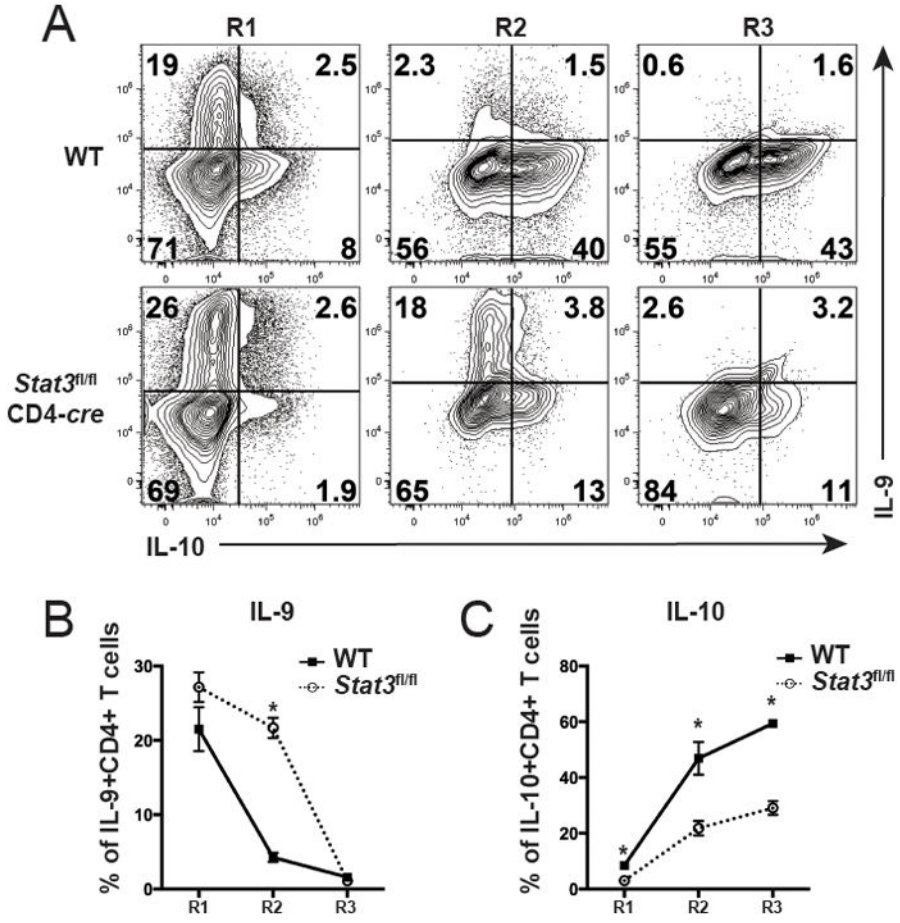

Figure 5.

The Th9 phenotype is maintained in the absence of STAT3. (A) Long-term Th9 cultures were preformed with *Stat3*$^{fl/fl}$ CD4-cre and wild type mice with evaluation of cytokines at D5 of each of the three rounds of differentiation. Representative dot plots show the intracellular cytokine-positive cells for IL-9 and IL-10 through the rounds of culture. (B) Frequencies of IL-9-secreting cells determined by flow cytometry quantified from (A). *p<0.05, significant difference between wild type and *Stat3*$^{fl/fl}$ CD4-cre IL-9-positive cells. (C) Frequencies of IL-10-secreting cells determined by flow cytometry quantified from (A). *p<0.05, significant difference between wild type and *Stat3*$^{fl/fl}$ CD4-cre IL-10-positive cells. These data are representative of 3 individual experiments with 3 individual mice per experiment with data presented in (B–D) as the mean ± SEM of three mice in one experiment.

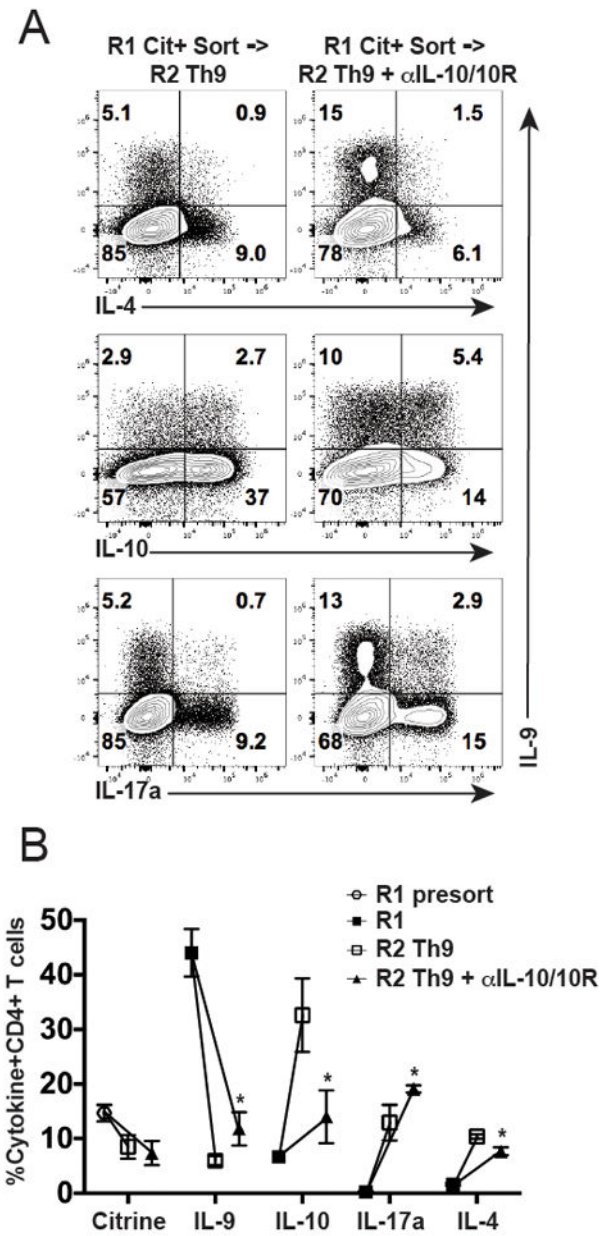

Figure 6.

IL-10R blockade increases stability of Th9 cells. (A) Th9 cells flow-sorted into (Cit+) populations were cultured in Th9 conditions with or without antibodies against IL-10 and the IL-10R receptor. Representative dot plots showing cytokine expression after stimulation with PMA/Ionomycin at the end of R2. (B) Frequencies of cytokine-positive cells originating from an R1 Citrine sort and the result of R2 with and without αIL-10/10R blockade. *$p<0.05$, significant difference between R2 Th9 with and without αIL-10/10R blockade. These data are representative of 3 individual experiments with 3 individual mice per experiment with data presented in (B–D) as the mean ± SEM of three mice in one experiment.